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## Strategies to decrease ongoing oxidant burden in chronic obstructive pulmonary disease

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### Abstract

Chronic obstructive pulmonary disease (COPD) is a leading cause of mortality and morbidity globally, and its development is mainly associated with tobacco/biomass smoke-induced oxidative stress. Hence, targeting systemic and local oxidative stress with agents that can balance the antioxidant/redox system can be expected to be useful in the treatment of COPD. Preclinical and clinical trials have revealed that antioxidants/redox modulators can detoxify free radicals and oxidants, control expression of redox and glutathione biosynthesis genes, chromatin remodeling and inflammatory gene expression; and are especially useful in preventing COPD exacerbations. In this review, various novel approaches and problems associated with these approaches in COPD are reviewed.

### Keywords

antioxidants; COPD; glutathione; Nrf2; oxidants; redox; thiol; tobacco smoke

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide either due to tobacco smoking or inhaled noxious gases, including smoke derived from biomass burning [1]. Its prevalence is escalating globally, especially in countries with high frequencies of smoking combined with significant environmental exposures to pollutants and biomass smoke [2]. In the Western world, COPD has been earlier estimated to be associated with smoking in over 90% of the cases, but this number may be significantly lower, as genetic background [3] and factors related to lung growth and development, maternal smoking, childhood infections, socioeconomic status, and a wide range of environmental exposures, such as ozone, nitrogen dioxide and car/diesel exhaust, also contribute to the development of airway disease and COPD [4]. Numerous studies and reviews have been recently published about COPD/emphysema, its development, pathogenesis, phenotypes, progression, systemic manifestations and therapies [5–11]. Smoking cessation is the major and most cost-effective way to prevent disease progression, but COPD can also develop in nonsmokers [12]. So far, no pharmacotherapy has been

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efficient enough to prevent ongoing inflammation/destructive processes in the lung, and inflammatory features associated with COPD subside very slowly after smoking has been discontinued [13–16].

The pathogenesis of COPD/emphysema is suggested to include oxidant–antioxidant and protease–antiprotease imbalance associated with innate immunity. Innate immunological factors in turn can lead to premature lung aging (senescence), apoptosis, decline in the immunological defense mechanisms and development of the disease [10,11,17–19]. These processes are either directly or indirectly associated with oxidative stress. Oxidant burden has multiple other consequences, such as induction of proteases, activation but also inactivation of defense mechanisms, and activation of growth factors and inflammatory genes [20,21]. Markers of oxidant burden have been detected by several techniques both in experimental models of smoke exposure and in human COPD [22–25]. Oxidant stress has been detected not only in the lung, but also in systemic circulation and in several other organs that are affected in COPD, further emphasizing the systemic nature of the disease [7,20]. COPD has been considered as a systemic inflammatory syndrome with multiple comorbidities [26]. This review will focus on the oxidant/antioxidant angle of COPD and on the cellular and molecular aspects of the lung, since the airways and the lung represent the first organ that is directly exposed to environmental toxins and cigarette smoke (Figure 1). However, even the results obtained from lung studies, for example on sputum, airway biopsies or lung tissues, need to be interpreted with a certain caution, since there are many COPD subphenotypes from airway-predominant to emphysema-predominant disease, and subtypes with or without symptoms of chronic bronchitis and sputum production. Moreover, the parenchymal lung disease, emphysema, is patchy, not evenly distributed, and may contain other pathological features besides emphysema, such as small-airway fibrosis and fibrotic lesions in the lung parenchyma. This review discusses the targets for antioxidant therapy, and various redox-mediated pharmacological options in the treatment of COPD.

## Major oxidant-producing mechanisms in the COPD lung

### Cigarette smoke as an inhaled oxidant

Cigarette smoke itself contains large amounts of free radicals and oxidants, more than  $10^{15-17}$  oxidant/free radical molecules per puff, so that the total number of highly reactive compounds in cigarette smoke is approximately 5000 [27]. The most important individual reactive compounds of cigarette smoke and/or tar include superoxide radical ( $O_2^-$ ), nitric oxide (NO), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\cdot OH$ ), quinones/semiquinones and ferrous compounds that can participate in numerous reactions that in turn produce other toxic and reactive metabolites in the lung. One of the toxic metabolites is peroxynitrite ( $ONOO^-$ ), which can diffuse to other cell compartments and locations. An additional problem with the semiquinone and quinone radicals is the fact that they contribute to continuous oxidant formation in the redox-cycling reaction in the cells [28].

### Enzymatic sources of oxidants in the COPD lung

The generation of reactive oxygen species (ROS) is highly elevated in lung cells, especially in smokers' lungs and in COPD. In normal situations low levels of ROS are critical in regulating numbers of cell signaling reactions by redox-state inducible reactions including antioxidant enzymes and related proteins. High levels of ROS/respiratory burst are primarily directed against exogenous bacteria and parasites, but they have, if the oxidant generation continues, a deleterious effect on cellular functions and on the development of lung injury. Typically oxidant generation is further enhanced and continued in smoking-related airway inflammation due to the activation of inflammatory cells. COPD is associated with neutrophilic airway inflammation [29], and these cells are one of the most potent producers

of free radicals/ROS [30]. Not only neutrophils, but also activated macrophages play a role in the pathogenesis of COPD [31]. Eosinophils, which are considered to be associated mainly with asthma, are also activated in COPD and are especially associated with COPD exacerbations and cases with some airway reversibility [32]. One additional feature in COPD is the accumulation of lymphocytes and lymphoid follicles in the small airways [33], with redox modulatory functions of cigarette smoke-mediated carbonylation [34]. The most important oxidant enzymes and/or pathways in these reactions include: NADPH oxidases, dual oxidases/peroxidases, myeloperoxidase, eosinophil peroxidase, nitric oxide synthases (NOS), xanthine oxidase and the mitochondrial electron transport chain [20,30]. COPD is considered a disease group, not only with several phenotypes, but it also shows more evident overlapping with asthma than previously expected [35].

## Oxidative biomarkers of COPD

Owing to the highly reactive nature of ROS and NO, their direct quantification is problematic and most studies have analyzed the levels of oxidative and/or NO-producing enzymes or footprints of oxidative damage (i.e., markers of oxidant-associated proteins, lipid and DNA modifications). These components have been analyzed/detected mainly in lung tissues, epithelial lining fluid, bronchoalveolar lavage fluid or sputum, and some others, such as NO, H<sub>2</sub>O<sub>2</sub>, hydrocarbons (such as ethane and pentane) and carbon monoxide, in the exhaled air or exhaled breath condensate [23–25,36]. Some of these components that have been used in the monitoring of oxidative stress in smokers/COPD are summarized below. Almost all ROS-generating systems, such as NADPH oxidase enzyme complex, dual oxidase/peroxidase, myeloperoxidase, NOSs, xanthine oxidase system and several peroxidases, have been found to be elevated in the lungs of patients with COPD [37,38]. Modification and reaction metabolites of ROS include lactoferrin, neutrophil lipocalin, chlorotyrosine (a product mainly from neutrophils), bromotyrosine (a product from eosinophils), markers of lipid peroxidation, such as malondialdehyde, 4-hydroxy-2-nonenal (4-HNE) and isoprostanes, and markers of protein nitration, protein carbonylation and DNA oxidation. Assessment of these markers has shown that there is remarkable oxidant burden and nitrosative stress in the COPD lung. Importantly, smoking alone can cause significant elevation in these compounds in human airways. For example, the levels of lipid peroxidation products such as 8-isoprostane are elevated in induced sputum and/or exhaled breath condensate both in smokers [39] and patients with COPD [40]. Another marker of lipid peroxidation, 4-HNE, is a highly reactive compound and elevated in the lung cells and lung secretions in smokers with airway obstruction [41], but also in current smokers who have not developed COPD compared with nonsmokers [42]. Many of these biomarkers have been used in the assessment of the severity of oxidative stress *in vivo*. Silencing these reactions, one example being inhibitors of NOS/ROS generation and/or activation of transcription factors associated with protective enzymes, have potential in preventing oxidative stress/progression of COPD.

Cigarette smoke can also cause formation of carbonyl adducts on cysteine, histidine and lysine residues on proteins. Aldehydes, which are formed during peroxidation of lipids can in turn modify proteins both in conjugation reactions, oxidation of amino acids and oxidative cleavage of proteins. Carbonylation and formation of oxidative stress-induced antibodies to carbonyl-modified proteins are known to occur in smokers and in subjects with COPD (Figure 1) [34,41]. Targeting protein carbonylation may be one potential way to prevent the progression of COPD.

Given that oxidant markers have also been detected in cigarette smokers without COPD, it remains unclear whether these compounds can be directly used in the assessment of COPD development, disease progression or activity, but it can be concluded that there is already

remarkable oxidative stress even in smokers who still have normal spirometry. The major oxidant markers detected in COPD are summarized in Table 1 [25]. It is increasingly clear that markers of oxidative damage are elevated in a variety of inflammatory lung diseases besides COPD, such as in asthma (however, there is significant overlapping between COPD and asthma), lung fibrosis (again in COPD there is airway fibrosis and occurrence of patchy fibrotic lesions in the emphysematous lung), lung infections and even in malignant lung diseases. It is demanding and it is unlikely that therapy will be developed based on only one oxidant marker detected in the COPD lung, since they can be associated with the disturbance of several different antioxidant and defense mechanisms in the lung.

## Major antioxidant enzymes & associated protective pathways in the lung

Antioxidants and antioxidant enzymes have a crucial role against oxidant burden in smokers/COPD lung. The redox sensitivity of the antioxidant enzymes vary; some are highly inducible, some constitutive, when the induction generally increases protection against oxidative stress. Enzymes are often induced by mild oxidant environment and cytokines, but the same enzymes can be inactivated by severe oxidative stress through: oxidation, nitrosylation, thiolation, glycosylation and/or proteolytic reactions by undergoing post-translational modifications. Knowledge of these mechanisms would significantly increase the understanding of key problems of the protective pathways that are of major importance when new antioxidant/redox-mediated strategies against COPD are being developed.

Lungs have efficient and highly specialized antioxidant capacity against exogenous free radicals and ROS [30,43,44]. Besides the classical antioxidant enzymes, there are many other proteins and molecules with significant antioxidant properties in the airways and lung parenchyma (e.g., mucins, which are cysteine-rich glycoproteins). In addition, epithelial lining fluid contains high levels of glutathione (GSH), a molecule that has been considered as one of the most important antioxidants in the human lung and airways [45]. Furthermore, lung and airways contain vitamins and several proteins that can bind free iron and other metals (e.g., albumin, transferrin, ferritin, ceruloplasmin and metallothionein), and thereby silencing oxidant generation. Classical human antioxidant enzymes (shown in Figure 2) have been widely studied; however, most original studies have focused on only one enzyme, not on their interactions, and the comprehensive understanding of their relative role in COPD has remained poorly understood.

## Superoxide dismutases

Superoxide dismutases (SODs) represent the only enzyme family with activity against superoxide radicals. These enzymes include copper–zinc SOD (CuZnSOD, SOD1), manganese SOD (MnSOD, SOD2) and extracellular SOD (ECSOD) [43,46]. SODs have highly specific locations in various compartments of lung cells and also extracellularly [47–49]. MnSOD is induced by cytokines such as TNF- $\alpha$  [50], oxidants and cigarette smoke [51], but MnSOD is also inactivated/oxidized by severe oxidative stress [52,53]. MnSOD deficiency is lethal, inducing toxicity to the CNS and heart, but in these circumstances the lung involvement is minimal [54]. Numerous studies have, however, confirmed the protective role of MnSOD against oxidants in the lung, although many studies have shown that the balance between superoxide and H<sub>2</sub>O<sub>2</sub> scavenging enzymes is more effective than elevation of only one enzyme (MnSOD). When compared with the results obtained with MnSOD, CuZnSOD is a less inducible, relatively stable, cytosolic bulk enzyme found in the airway epithelium [43,47]; however, its overexpression has been shown to attenuate inflammatory response against cigarette smoke [55]. ECSOD is induced by cytokines instead, although to a lesser degree compared with MnSOD [56]. ECSOD-deficient mice

develop relatively normally, but when they are exposed to high oxygen tension their survival is shortened [57]. In agreement, ECSOD overexpression protects against oxidants and inflammation, and in experimental studies its deficiency leads to emphysema [58], suggesting an essential role of ECSOD in protecting against COPD [59,60]. In summary, MnSOD participates in the protection of the lung periphery/alveolar epithelium, and ECSOD protects the lung parenchyma, although their specific significance in COPD is still poorly understood.

## H<sub>2</sub>O<sub>2</sub> & peroxide scavenging pathways

The most important 'classical' antioxidant enzymes associated with H<sub>2</sub>O<sub>2</sub> decomposition include catalase and enzymes associated with GSH homeostasis. The reactivity of these enzymes varies at different oxidant circumstances and cell organelles. Catalase is poorly inducible; it is highly reactive at high oxygen tension, but only against H<sub>2</sub>O<sub>2</sub>, and located mainly in peroxisomes of the lung inflammatory cells and alveolar epithelial type II cells [61]. Based on experimental studies, the best lung protection has been obtained when both SOD and catalase are simultaneously provided [62]. It is therefore likely that manipulation of catalase alone or giving a mimetic with only catalase activity is not efficient in protection against oxidative stress in COPD.

GSH is one of the most important nonenzymatic antioxidants in human airways [45], and in normal situations is mainly (>90%) in the reduced form. GSH homeostasis is linked to a large network of enzymes [44]; GSH peroxidases (GPXs) decompose both H<sub>2</sub>O<sub>2</sub> and lipid peroxides in GSH-requiring reactions that oxidize GSH to GSSG, which in turn is reduced by glutathione reductase. The rate-limiting enzyme in GSH biosynthesis from amino acids is glutamate–cysteine ligase (GCL), which contains two differentially regulated subunits. Another enzyme in the GSH synthesis is glutathione synthase.  $\gamma$ -glutamyl transpeptidase is a plasma membrane enzyme forming glutamylcysteine for GSH synthesis [63]. GCL, GPXs and the glutathione-S-transferase family of enzymes, such as GST- $\alpha$ , - $\pi$ , - $\mu$  and - $\omega$ , have been characterized in human lung and found to be expressed mainly in airway and alveolar epithelium, and in airway secretions [64–66]. Glutaredoxins (GRX I and II) are thiol disulfide oxidoreductases with antioxidant and catalytic functions and are involved in intracellular and extracellular homeostasis of glutathionylated proteins and GSH in the lung [67], and are positively regulated by NF- $\kappa$ B [68]. In human lung, GRX1 is expressed mainly in alveolar macrophages and airway epithelial cells [69]. Exposure to cigarette smoke leads to decreased *GRX1* mRNA and protein along with decreased activity and increased protein S-glutathionylation [70]. Hence, strategies to increase both GSH and *GRX1* gene expression would have the potential to control cigarette smoke-induced diseases, such as COPD.

Most GSH-related antioxidant enzymes are induced by Nrf2-mediated mechanisms. Nrf2 is a basic-leucine zipper transcription factor present in the cytoplasm of normal cells. In response to oxidative and electrophilic stresses, Nrf2 detaches from its cytosolic inhibitory subunit Keap1 and translocates into the nucleus, wherein Nrf2 binds to the antioxidant response element (ARE) of target genes along with other binding factors, leading to the induction of stress response genes [71–73]. Nrf2 regulates almost all antioxidant enzymes and Phase II cytoprotective genes that regulate GSH maintenance such as: GCL modifier subunit, GPXs, several members of the GST family, heme oxygenase-1 (HO-1) and NAD(P)H/quinone oxidoreductase 1 (NQO1) [74,75]. Studies with *Nrf2*-null mice have shown greater susceptibility of these mice to cigarette smoke-induced emphysema compared with wild-type mice [76,77], thus suggesting the protective role of Nrf2 against COPD/emphysema development (Figure 3).



## Thioredoxin–peroxiredoxin–sulfiredoxin pathway

Thiol-containing proteins thioredoxins (TRX1 and TRX2) and thioredoxin reductases belong to the oxidoreductase family of redox sensors participating in  $H_2O_2$  disposal, cell proliferation and resistance to apoptosis [78]. These enzymes have been detected in human lung [79] but also in lung malignancies [80]. TRX is primarily bound to proteins, such as hepatopoietin [81] and ASK-1. While overexpression of TRX1, primarily due to its antioxidant property, attenuates cigarette smoke-mediated oxidative stress and emphysema [82], this effect in cigarette smoke-mediated lung damage and both in the pathogenesis of COPD and lung cancer remains to be investigated.

Peroxiredoxins (PRXs) belong to a superfamily of selenium-independent (mainly TRX) peroxidases. Six different PRXs have been found in human cells, classified as typical double-cysteine (PRDX1–PRDX4), atypical double-cysteine (PRX5), and single-cysteine (PRX6) classes, depending on their number of active Cys residues [83]. They have powerful peroxidase activity against  $H_2O_2$ , peroxynitrite and phospholipid hydroperoxides [84]. Normal human lung expresses all six PRXs, both intra and extra-cellularly [85,86], but the levels are even higher in lung and pleural malignancies [87,88]. A series of studies involving *PRX1* gene knockout mice have shown a protective role of PRX against oxidative stress [89]. Another study hypothesized that targeted disruption of *PRX6* would lead to susceptibility to cigarette smoke-mediated lung inflammation and/or emphysema in mouse lung. Importantly, the study suggested a critical role of lung PRX6 combined with several compensatory mechanisms during acute cigarette smoke-induced adaptive response [90]. These results underline the importance of antioxidant compensatory mechanisms against exogenous oxidants. Based on the results, strategies to increase the expression or pharmacological activation of PRX by small molecules may be effective in the treatment of COPD.

Sulfiredoxins (SRX) represent a family of cysteine-containing proteins that play a key role in protection against oxidative stress, having the capability to maintain the balance between  $H_2O_2$  production and elimination and protection against apoptosis [91]. These enzymes catalyze the reduction of cysteine sulfinic acid to sulfenic acid, which can restore the activity of overoxidized PRXs [91–94]. SRX1 is expressed in human lung especially in macrophages [95], and induced in the lungs of Nrf2-overexpressing mice in response to cigarette smoke [96], further suggesting its importance against oxidative stress related to smoking and COPD.

## Levels & activities of the antioxidant enzymes, related proteins & Nrf2 in smokers & in the COPD lung

A microarray study was conducted on bronchial brushings using 44 antioxidant-related genes in four categories (catalase, SOD/GSH metabolism and pentose phosphate cycle) in nonsmokers and smokers who had no diagnosed lung disease [97]. A remarkable interindividual variability could be seen; GSH-associated enzymes and SOD3 in particular were significantly induced by cigarette smoke in human lung *in vivo* [20]. However, based on this study the significance of these changes in the development of COPD remains unclear. Only a small number of individuals who smoke finally develop COPD and the induction is not a marker of disease development. There is another corresponding study, which also investigated airway bronchial brushings and gene expression of oxidant/antioxidant genes using Affymetrix technology in nonsmokers, smokers and subjects with variable COPD severities [98]. The major findings of this study suggest distinct nonlinear gene expression patterns across the various COPD severities. Some enzymes (e.g., SOD2) were upregulated in smokers with risk for COPD (previous stage 0 COPD in GOLD

classification [8]), some others were downregulated possibly due to adaptation, while some were also elevated with increasing COPD severity. Overall, these studies underline the complexity of the antioxidant/oxidant-related pathways in human airways *in vivo*. The Hackett *et al.* study was on gene expression, which does not necessarily correlate with protein levels, protein modifications or enzyme activity. MnSOD is elevated by smoke exposure [51], in smoker's lung and mild COPD [99], while no change has been seen in the total SOD activity or in the expression of CuZnSOD [99]. ECSOD protects against emphysema via reduction of oxidative extracellular matrix fragmentation and oxidative post-translational modifications of elastin fragments (leading to autoantibody production) in the lung of cigarette smoke/elastase-induced mouse models of emphysema [58]. Results on human COPD have shown that there is no apparent change in the ECSOD immunoreactivity in mild/moderate COPD, but the immunoreactivity declines in specific locations of the lung in very severe COPD [100]. The development of pharmacological mimetics to replenish/augment SOD3 could be a rational therapeutic intervention for COPD/emphysema.

Loss of Nrf2-positive regulator DJ-1 and post-translational modifications of the Keap1–Bach1 equilibrium leads to the downregulation of Nrf2 (GSH levels) in pulmonary macrophages and in the lungs of patients with COPD [76,101–104]. Recent studies have reported that Nrf2 protein level is significantly decreased (by oxidative post-translational modifications) both in the lung tissues and in alveolar macrophages of patients with COPD/emphysema with a parallel decrease in HO-1, GPX2 and NQO1 along with elevated levels of Keap1 and Bach1 [101]. This finding has been confirmed in another study where the lungs of the patients with COPD showed significant decline in Nrf2-associated detoxification enzymes, GSH and DJ-1, a protein that is a Nrf2 stabilizer [102], further emphasizing the association of Nrf2 decline with COPD.

Results on extracellular versus intracellular GSH level/homeostasis vary. GSH is increased in bronchoalveolar lavage fluid of smokers [44,105], but not in the cells of the bronchoalveolar lavage fluid (mainly alveolar macrophages) of elderly smokers or subjects with COPD. GPX 3 is increased during oxidative stress of human airways and/or both in the bronchial epithelium and epithelial lining fluid of smokers [106], but its RNA levels decrease in severe COPD [107]. *GCL* mRNA expression appears to elevate in the bronchial epithelium in COPD [108] while lowered GCL immunoreactive protein in the bronchial epithelium and alveolar macrophages in COPD has been reported [109]. Interestingly, GCL immunoreactive protein has been shown to increase in certain cell types (i.e., metaplastic/dysplastic epithelium in the COPD lung), a corresponding finding also being detected with some other oxidant-regulated enzymes [110]. These studies emphasize the complex role of oxidant stress and antioxidant enzymes, and the critical role of their ideal balance in lung/bronchial epithelial cell survival, proliferation, carcinogenesis and/or cell death.

GSTs and HO-1 are highly inducible and potentially protective enzymes against smoke-induced inflammation. The mRNA of several GSTs is elevated in the bronchial epithelium of smokers without COPD [97], but lung immunoreactivity of several GSTs and possibly GRX1 declines in severe COPD [66,111,112]. Similarly, HO-1 is elevated in the alveolar macrophages of smokers, but is decreased in severe COPD [113,114]. Several members of the TRX/PRX family either remain unchanged or increase in COPD and appear to be relatively resistant against oxidative stress *in vivo* [97,115,116]. However, SRX, which is capable of reversing PRX over-oxidation, declines in very severe COPD [96]. Collectively, these studies suggest that not only several antioxidant enzymes, but also SRX levels, are decreased with increasing COPD severity, which in turn are additional reasons to pursue therapeutic strategies to maintain their ideal balance in COPD.



## Antioxidant/redox-modulating therapies to dampen oxidant burden in the lung

The most important and cost-effective way to prevent COPD progression and to decrease oxidative stress is smoking cessation [20]. This is highly important since, so far, there has been no pharmacological therapy that has a clear prognostic significance in COPD. Other environmental exposures besides smoking can contribute to COPD development, but prevention of all these exposures is practically impossible. In addition, several studies have emphasized the problem of ongoing inflammation, oxidant and protease burden, months and even 1–2 years after smoking cessation [13–16], which can enhance disease progression. Inhaled corticosteroids are highly effective in treating asthma, but the problem is that in COPD their role is weak, being beneficial only in cases with asthma and COPD overlapping and in those COPD patients who have repeated exacerbations [8]. One mechanism that is associated with steroid resistance is oxidation/*S*-nitrosylation of HDAC2 at Cys-262 and Cys-274 [117]. By contrast, statins, which have also systemic anti-inflammatory and redox modulatory effects, have been found to decrease both COPD exacerbations and mortality [118,119]. Since one of the causes of COPD is due to the generation or exposure to oxidants, it is possible that the disease process may be controlled either by downmodulating ROS generation or by therapeutic intervention with one or more antioxidants/redox modulators [7]. It is important to note that COPD has several clinical subphenotypes [120]; it will, therefore, be appropriate to map which type of oxidants are associated with a particular phenotype that will aid in selecting a specific antioxidant for a specific subphenotype. Administration of antioxidants for therapeutic purposes may be achieved either, by increasing the endogenous antioxidant enzyme defenses or by enhancing the nonenzymatic antioxidant levels via pharmacological systemic and/or inhaled routes.

### Small molecule thiol antioxidants

#### N-acetyl-L-cysteine

*N*-acetyl-L-cysteine (NAC) is an acetyl derivative of the amino acid cysteine and is a strong reducing agent (Box 1). NAC is a mucolytic agent that improves mucociliary clearance. It is deacetylated into cysteine in the GI tract and serves as precursor of GSH in cells. Oral administration of NAC 600 mg twice daily for 2 months was associated with a reduction in bronchial hypersecretion and a decline in FEV<sub>1</sub> and exacerbations [121]. However, clinical studies regarding the beneficial effects of NAC and other thiols in patients with COPD have yielded mixed results (Table 2) [121–129]. While a Cochrane systematic review showed a significant reduction of exacerbations compared with placebo, a small-scale trial failed to demonstrate any clear clinical benefits [123]. A few meta-analyses, however, have shown a small, but significant clinical benefit in COPD [126]. Overall, NAC may be more likely to be of benefit in subjects with mucus hypersecretion in specific COPD phenotypes and COPD exacerbations.

#### N-acystelyn

*N*-acystelyn (NAL), a lysine salt of NAC, is a mucolytic and antioxidant (reducing) thiol compound (Box 1). NAL has a neutral pH in solution and can be directly aerosolized into the lung with virtually no side effects [130]. Therefore, NAL may be a potential therapeutic candidate for COPD treatment and a clinical trial using NAL for treatment of COPD is warranted.

#### Erdosteine

Erdosteine is a mucoactive thiol antioxidant (Box 1) that can also reduce bacterial adhesiveness. The drug acts by breaking the disulfide bonds of mucus glycoproteins,

affecting the physical properties of the mucus, thus leading to increased cough clearance [131]. EQUALIFE, a randomized, placebo-controlled clinical study involving oral administration of 300 mg erdosteine twice daily for 8 months [128], showed a significant reduction in COPD exacerbations compared with the placebo group. In another study, administration of erdosteine 300 mg twice a day for 7–10 days improved symptoms and recovery period from acute exacerbations of COPD [132]. Furthermore, long-term treatment of stable COPD patients with erdosteine reduced both hospitalizations and acute exacerbations. Erdosteine has been reported to impart health benefits in patients suffering from repeated, prolonged or severe exacerbations of COPD [132,133]. Overall, erdosteine has been widely introduced in the clinic, especially in patients with COPD exacerbations.

### Carbocysteine

*S*-carboxymethylcysteine (carbocysteine or S-CMC), with mucoactive, antioxidant and anti-inflammatory properties, is a thiol derivative of the amino acid *L*-cysteine (Box 1). This compound inhibits kinins and prevents bronchial inflammation and bronchospasm [134]. Due to its ability to reduce bacterial respiratory tract infections in COPD, it has been suggested that carbocysteine may act via inhibition of pathogen adherence to cells [135,136]. Some reports of carbocysteine in the treatment of COPD patients are available (Table 2) [129,136–143]. The PEACE study investigated the effect of treatment of 709 Chinese COPD subjects for 3 years with carbocysteine (250 mg three times daily) and concluded that COPD patients treated with carbocysteine experienced fewer exacerbations per year [129]. More systematic studies are required to understand the detailed mechanism of action of carbocysteine and to emphasize the candidature of carbocysteine as a major therapeutic agent for treatment of COPD.

### Fudosteine

Fudosteine ([(-)-(R)-2-amino-3-(3-hydroxypropylthio)] propionic acid) (Box 1) has been used in the treatment of chronic respiratory diseases, such as bronchial asthma, chronic bronchitis, COPD and bronchiectasis, as a mucoactive agent [144,145]. It has greater bioavailability than NAC and acts by elevating the cysteine levels in the cells. Fudosteine inhibits mucin hyper-secretion by downregulating *MUAC5AC* gene expression [144]. Fudosteine has shown to have a promising potential in treatment of patients with COPD; however, much is not known about its mode of action [146].

### Other thiol compounds

Procysteine (*L*-2-oxothiazolidine-4-carboxylate or cysteine *L*-2-oxothiazolidine-4-carboxylic acid) is a cysteine-relieving compound that increases the intracellular cysteine levels, but has no established role in the clinic. *N*-isobutyrylcysteine has similar effect as NAC, but with greater bioavailability. However, therapeutic evaluations of *N*-isobutyrylcysteine in COPD have not been better than placebo [130,147]. Ergothioneine (2-mercaptohistidine trimethylbetaine) is a naturally occurring antioxidant found in most plants and animal tissues (Box 1) [148] and is chemically a betaine of 2-thio-*L*-histidine. The therapeutic efficacy of ergothioneine is enhanced by its ability to increase cellular tolerance/availability of *N*-acetyl-*L*-cysteine. Thus, ergothioneine may be a potential antioxidant/anti-inflammatory agent to modulate chronic inflammatory lung diseases including COPD. Overall, therapeutic strategies involving GSH or its analogs or thiol compounds must be carefully and cautiously designed keeping in mind the species barrier, bioavailability, half-life of the analogs, generation of toxic byproducts by the analogs and interference with metabolic and signaling pathways.

The clinical trials, Cochrane analyses and meta-analyses for the efficacy of various thiol antioxidants in COPD have been summarized in Tables 2 & 3.

## Nrf2 activators

Compounds that activate Nrf2 or stabilize Keap1/DJ-1/Maf proteins may have a crucial role, especially in situations where the endogenous antioxidant system is weakened (one example being severe–very severe COPD) or is less adaptive/compensatory or declined (e.g., during aging). Dietary and synthetic compounds such as sulforaphane [149], dithiolethione [150], curcumin and caffeic acid phenethyl ester [151] can induce ARE-regulated gene expression and have been shown to exert chemopreventive activities. Furthermore, activation of Nrf2 by sulforaphane (present in broccoli and cruciferous vegetables) leads to HDAC2 denitrosylation (not known whether it affects the activity and/or levels), restoring dexamethasone sensitivity [117], which suggests that Nrf2 activators may function as an additional tool in minimizing corticosteroid resistance in COPD.

Chalcones (1,2-diphenyl-2-propen-1-ones) and Michael acceptors are important groups of naturally occurring compounds [152] and have the ability to bind to proteins related to apoptosis and proliferation [153]. Chalcones can impart anti-inflammatory effect due to their ability to inhibit the NF- $\kappa$ B pathway [154] and simultaneously activate the Nrf2/ARE pathway, thus inducing the expression of Phase II detoxifying enzymes. Currently, various derivatives of chalcones are being developed for improving the anti-inflammatory and anticancer properties of these compounds for potential therapeutic roles in COPD [155].

## Lipid peroxidation & protein carbonylation inhibitors/blockers

### Edaravone (MC-186)

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free-radical scavenger that exhibits its antioxidant ability by inhibiting lipid peroxidation and protein carbonylation [156–158]. In one study, edaravone decreased infiltration of neutrophils and membrane lipid peroxidation and downregulated expression of IL-6 mRNA in the lungs with significant reduction in mortality [159]. Further studies are required to confirm the therapeutic efficacy of edaravone in the management of COPD and its symptoms [160–162].

### Lazaroids (U75412E or tirilazad mesylate): nonglucocorticoid 21-aminosteroids

Lazaroids (21-aminosteroid, U75412E) are a group of nonglucocorticoid analogs of methyl-prednisolone that can penetrate hydrophobic regions of the cell membrane, preventing peroxidation of membrane lipids [163,164]. Further studies are required to evaluate the efficacy of lazarooids as a therapeutic strategy in COPD.

### Other protein carbonylation inhibitors/blockers

Apart from the above approaches, an additional approach to reverse protein carbonylation would be the activation of enzymes involved in the reversal of protein carbonylation [160–162]. Such compounds would be diethylstilboestrol and 2,2'-dithiodipyridine. Their role in COPD awaits future investigation.

## Antioxidant enzyme mimetics & spin traps

### SOD mimetics

SOD mimetics can be classified into three categories. The first class includes several manganese-based macrocyclic ligands, such as M40401, M40403 and M40419 [165,166]; the second class are manganese-metaloporphyrins, such as AEOL-10113 and AEOL-10150 [167,168] and the third category are 'salens' (manganese-based SOD mimetic). Salens have an additional advantage as they have also been reported to have catalase-like activity and therefore, capability in neutralizing H<sub>2</sub>O<sub>2</sub> along with their ability to decompose toxic ONOO<sup>-</sup> [169]. So far only the second class of SOD mimetics have been studied in

experimental models of airway inflammation [165], suggesting that these compounds have significant antioxidant enzyme properties and may be used as novel anti-inflammatory drugs in airway diseases including COPD.

Broad antioxidant properties and capability to scavenge superoxides, lipid peroxides,  $\text{ONOO}^-$  and  $\text{H}_2\text{O}_2$  have been attributed to the metalloporphyrin-based catalytic antioxidant, manganese (III) meso-tetrakis-(*N*-methypyridinium-2-yl) porphyrin (MnTE-2-PyP) [170–172]. Administration of MnTE-2-PyP decreases inflammation and injury induced by a wide variety of factors [173–175]. Administration of another SOD/catalase mimetic EUK-189 improves redox status and decreases oxidative injury [176] and GSH/GSSG ratio [177]. These compounds may have potential for therapeutic use in COPD, but require further investigation.

### Glutathione peroxidase mimetics

Ebselen, a selenium-based organic complex, mimics the activity of GPX, thereby increasing the efficiency of GSH. BXT-51072 and BXT-51077 are low-molecular-weight, orally active, organoselenium GPX mimetics that possess peroxide neutralizing capabilities and prevent activation of inflammatory mediators. Inhibition of transcription of the inflammatory mediators is probably the underlying mechanism for the protective effects of ebselen. So far no reports are available on their protective effect against cigarette smoke-induced lung inflammation.

### TRX mimetics

Inhibiting TRX in the nucleus with MOL-294 (a low-molecular-weight inhibitor of TRX) blocks nuclear activation of both NF- $\kappa$ B and AP-1-dependent transcription [178]. Similarly, inhibition of Ref-1, another redox sensor, by PNRI-299 leads to the inactivation of the AP-1 pathway [179]. Recently, a family of triand tetra-oligopeptides, derived from the canonical CxxC motif of the TRX active site and a modified CxC motif, has been synthesized [180]. These TRX mimetics have been shown to lead to the upregulation of various redox-sensitive processes in the nucleus [180]. Thus, upregulation of TRX by various synthetic small molecules has been suggested to have therapeutic potential for the treatment of airway diseases including asthma and COPD.

### Peroxynitrite decomposition catalysts

Peroxynitrite decomposition catalysts (PDCs) are iron-containing porphyrin complexes with very similar structures to AEOL10150 and AEOL10113. Unlike other enzyme mimetics, PDCs possess catalytic activity against peroxynitrite [181,182]. The efficacy of PDCs has been reported *in vivo* in animal models that are associated with peroxynitrite generation [183]. Whether or not this class of antioxidants is also effective in COPD, a disease that is known to be associated with high levels of peroxynitrite generation, needs further investigation.

### Spin traps

Spin traps are chemical agents that can quench free radicals to form detectable stable end products and are as such used for studying reactions involving free radicals. The disadvantage of earlier spin traps included extremely short half-lives and their generation of dangerous hydroxyl radicals on decay. This problem has been overcome by introduction of electron-withdrawing moieties around the core pyrroline ring [184]. Phenyl-base nitron spin trap derivatives, such as NXY-059 (phenyl-base nitron spin trap-2,4-disulfonate), have been shown to impart therapeutic benefits in a wide variety of animal models of lung diseases, but they have not yet been tested in COPD.

## NOS inhibitors

Scavenging of NO can be used in reversing *S*-nitrosylation of HDAC2, which occurs in patients with COPD [117,185,186]. However, this reversal of HDAC2 may not work if HDAC2 is carbonylated by cigarette smoke. Nevertheless, further investigations are required to develop these compounds for their long-term use in oxidative/carbonyl stress-mediated chronic lung diseases, in particular in patients with COPD with steroid resistance. Recent experimental studies have suggested that inhibition of iNOS by various chemical inhibitors (N(6)-(1-iminoethyl)-L-lysine [L-NIL], G-nitro-L-arginine-methyl ester or L-NAME) results in attenuation of emphysema [187,188]. It is possible that NOS inhibition has side effects, and this is why selective inhibition of NOSs combined with that of other antioxidants should be carefully evaluated in the future.

## Literature search

The literature for this review was gathered by PubMed search [201] using specific keywords, such as cigarette smoke and/or COPD, oxidants, antioxidants and antioxidant enzymes. For therapeutic sections and drug groups on Cochrane analysis, keywords such as COPD therapeutic agents, antioxidant mimetics, antioxidant enzymes and Cochrane analysis from previously evaluated/published papers were used. Most original studies that have been published before the year 1990 have been excluded according to the journal instructions.

## Expert commentary

Oxidative/carbonyl stress is one of the major factors associated with the pathogenesis of COPD. Even though some earlier studies on antioxidant molecules directed against oxidative stress in COPD have been disappointing, it is even more important to find better, more specific, safe and efficient redox-modulatory compounds that can be directed to COPD since the disease itself is heterogeneous with variable subphenotypes from airway inflammation to emphysema. Antioxidant molecules that have been tested earlier in human studies have often been nonspecific with some possessing pro-oxidative side effects *in vivo*, an aspect that needs careful evaluation. In addition, previous studies have included subjects with COPD as one single group, even though COPD is a complex disease entity containing several different subphenotypes. Furthermore, more severe cases of COPD are associated with comorbidities, such as ischemic heart disease, metabolic syndrome, diabetes, osteoporosis and lung cancer; and these problems have generally not been evaluated in those earlier studies. One group of patients with COPD had exacerbations, again a patient group that suffers not only from COPD alone, but also from viral and bacterial infections and cardiac problems with worsened prognosis [8]. Studies on redox modulators/antioxidant molecules should include carefully evaluated and characterized groups of COPD patients with specific subtypes of the disease where the abovementioned problems have been taken into consideration.

Besides problems with the variety of subphenotypes, the oxidant/antioxidant pathways in human lung are also complicated. Earlier studies have not been systematic – that is, the importance of the key protective pathways or compensatory mechanisms in lung protection were not understood. A better understanding of these pathways, their relative importance in the airways versus parenchyma and the reality that AOE are also associated with lung and pleural malignancies [189,190], are of essential importance when new therapeutic redox modulators are being evaluated. Much has been done; for example, several recently discovered mechanisms and developed antioxidants/redox modulators are ideal molecules for testing specific key reaction cascades in the lung [7,17,20,24,25].



## Five-year view

It is likely that COPD, for example, airway/chronic bronchitis subtype, parenchymal damage/emphysema subtype or a disease with frequent exacerbations, react differently to specific redox modulator therapies and antioxidant mimetics – that is, the potential drugs may have different effects in different types of disease. Careful subphenotyping of COPD is realistic and several potential mimetics have already been developed. In addition, achievements to better understand various antioxidant enzyme pathways of the lung have been remarkable and several redox modulators have already succeeded in restoring, for example, SOD/H<sub>2</sub>O<sub>2</sub> balance and GSH-associated pathways. Many of them are directly/indirectly linked to Nrf2-mediated pathways, which in turn can protect the lung against oxidants and peroxidation/carbonylation reactions, thus preventing disease progression. Testing of their relative significance in COPD without overlooking their possible effects on cell survival and carcinogenesis, is of major importance. Antioxidant compounds may also enhance the efficacy of glucocorticoids by quenching endogenous oxidants and aldehydes in COPD. Furthermore, the detailed studies on antioxidants/redox modulators need to be tested, not only alone, but also in combination with anti-inflammatory drugs, antibiotics, statins and other therapies.

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## Website

201. PubMed search engine. [www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)

**Box 1. List of thiol antioxidants and Nrf2 activators****Antioxidants****Thiols:**

- *N*-acetyl-L-cysteine
- Erdosteine
- Fudosteine
- Carbocysteine

**Nrf2 activators**

- Sulforaphane
- Curcumin

**Triterpenoids:**

- CDDO

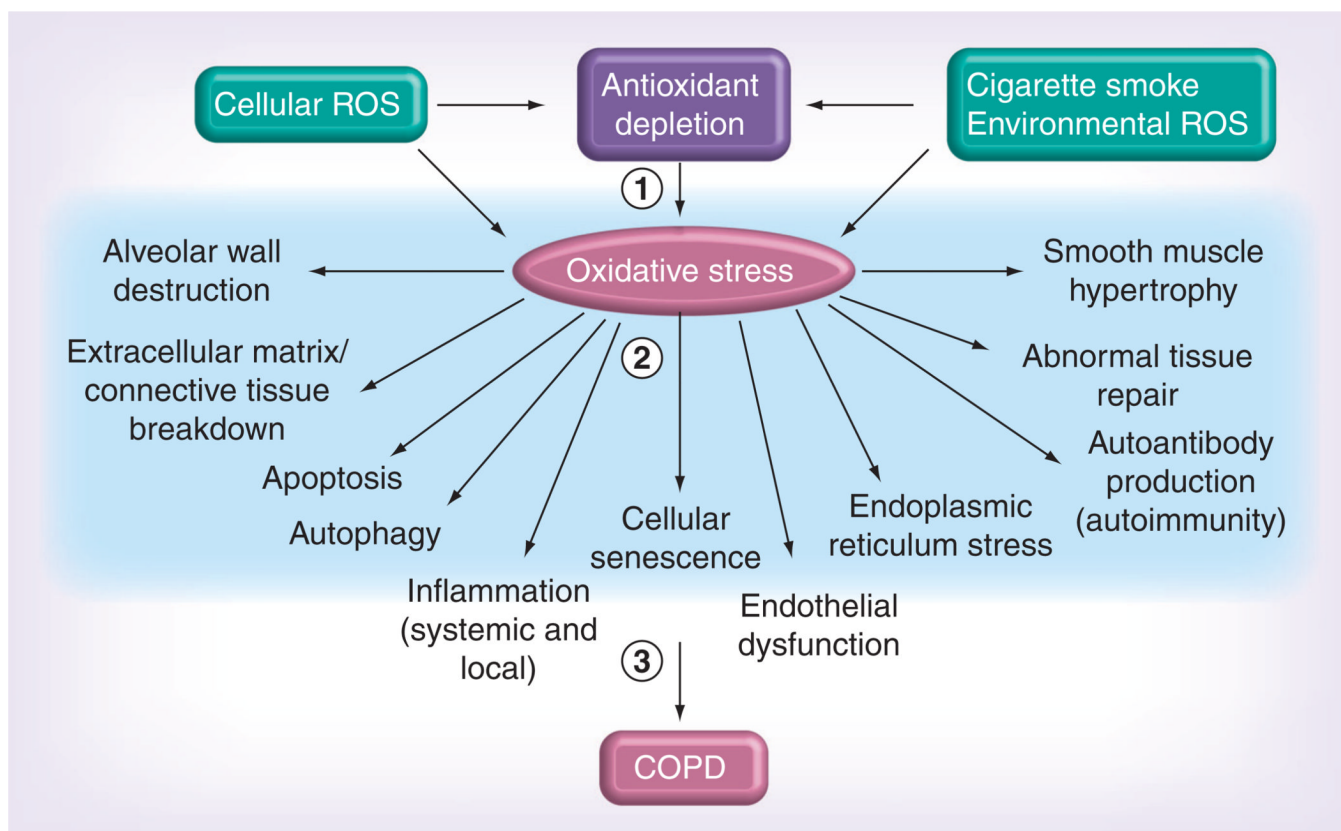
**Other analogs of CDDO:**

- CDDO-Im
- Dihydro-CDDO-trifluoroethyl amide
- CDDO methyl amide

CDDO: 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Im: 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole.

**Key issues**

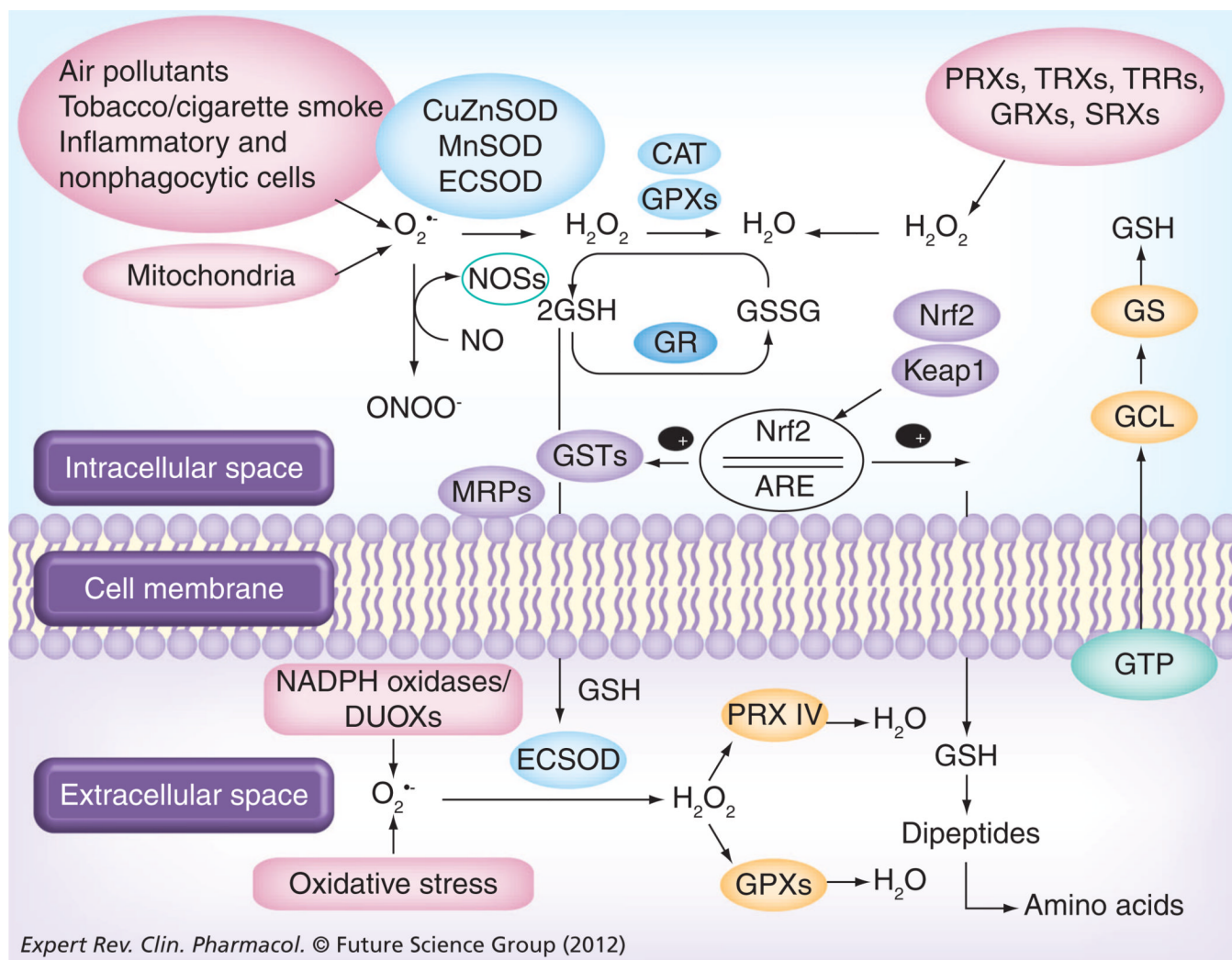
- Chronic obstructive pulmonary disease (COPD) is a devastating disease with several phenotypes and systemic manifestations.
- The pathogenesis of COPD is associated with oxidant burden.
- Oxidant–antioxidant imbalance is related to inhaled noxious compounds, activation of oxidant-producing enzymes and decline in antioxidant defense.
- One major issue is to find ideal compounds that dampen the specific oxidant-producing pathways and strengthen the endogenous vital antioxidant defense in the lung.
- Synthetic compounds with antioxidant/redox-modulating capabilities have been developed, some of them being already implemented in clinical practice.
- The newest molecules, such as ECSOD mimetics, Nrf2 activators and nitron spin traps, will require careful testing in various COPD subphenotypes.



**Figure 1. Consequences of oxidative stress in chronic obstructive pulmonary disease**

The pathogenesis of COPD involves several oxidative stress-induced cellular and molecular processes. Oxidative stress imposed by inhaled toxicants or produced by endogenous sources can lead to depletion of lung antioxidants (step 1). Oxidant/antioxidant imbalance in favor of oxidants can lead to abnormal activation of various cellular and biochemical processes, which can lead to various cellular and biochemical/molecular events (step 2) involved in the pathogenesis of COPD (step 3).

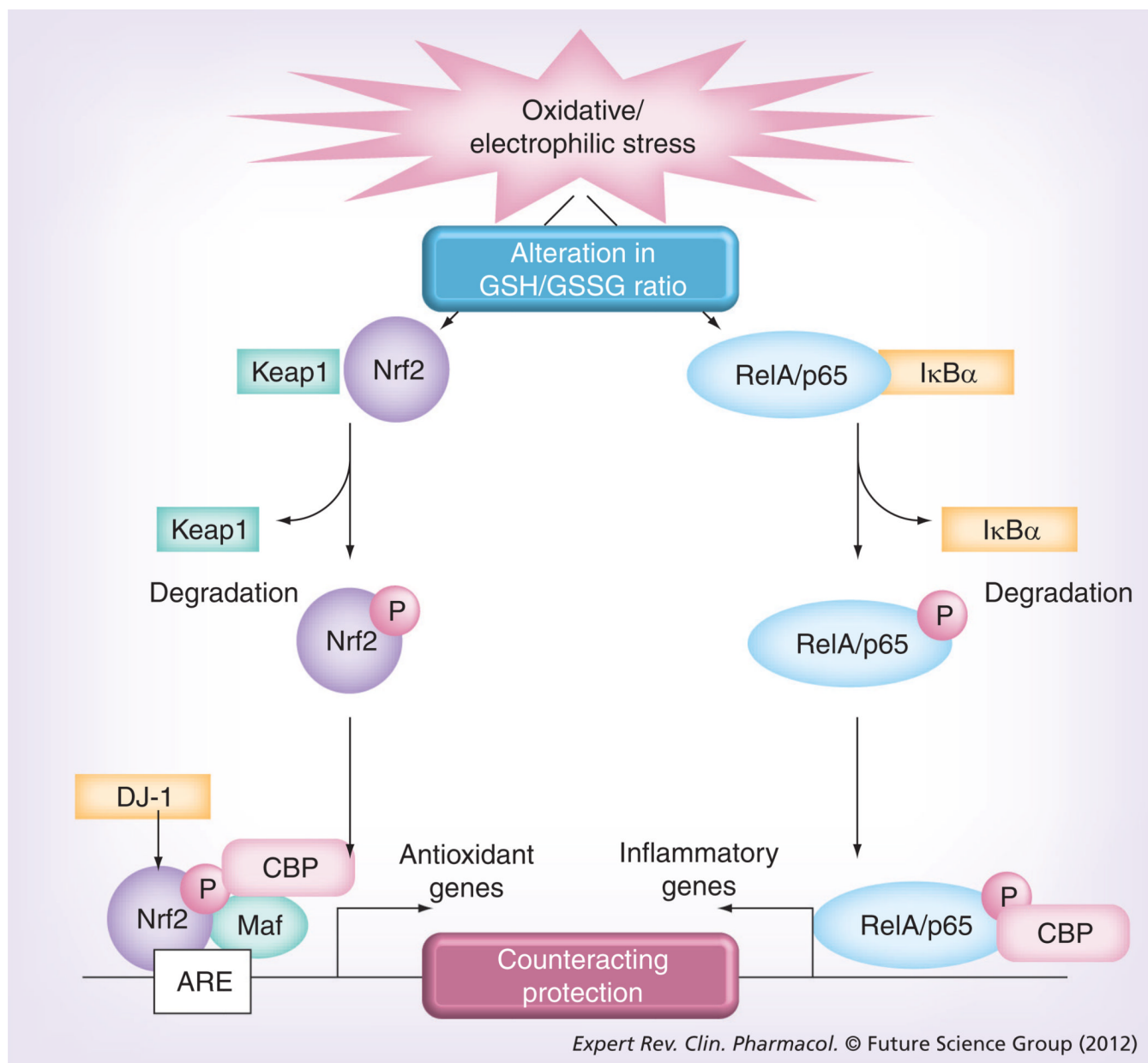
COPD: Chronic obstructive pulmonary disease; ROS: Reactive oxygen species.



**Figure 2. Schematic pathway diagram showing some of the major antioxidant enzymes and their interactions in the lung**

ARE: Antioxidant response element; DUOX: Dual oxidase;  $H_2O_2$ : Hydrogen peroxide; NO: Nitric oxide; NOS: Nitric oxide synthase;  $O_2^{\cdot-}$ : Superoxide radical;  $ONOO^-$ : Peroxynitrite.





**Figure 3. Nrf2 and NF-κB: antioxidant and anti-inflammatory counteracting responses**

Oxidative/electrophilic stress activates NF-κB, leading to induction of proinflammatory genes via histone acetylation/modifications (binding of cofactor CBP on promoters of proinflammatory genes). Oxidative/electrophilic stress can also activate the Nrf2 transcription factor, leading to its interaction with other small maf proteins culminating in various antioxidant Phase II gene transcription. Activated Nrf2 also interacts with CBP coactivator in the nucleus. This Nrf2–CBP interaction deprives the nuclear pool of CBP for its interaction with NF-κB, and hence results in overwhelming antioxidant response, or counteracting the inflammatory response. DJ-1 can stabilize Nrf2. ARE: Antioxidant response element; GSH/GSSG: Reduced glutathione/oxidized glutathione; P: Phosphorylation.

**Table 1**

Detected biomarkers of oxidative stress in patients with chronic obstructive pulmonary disease.

Biological samples	Biomarker	Outcome	Ref.
Exhaled air/breath condensate	Exhaled nitric oxide; hydrogen peroxide; volatile organic compounds; ethane; pentane; F2-isoprostanes	Elevation compared with controls	[25]
Sputum/biopsies	Activated neutrophils; dual oxidases/peroxidases; xanthine oxidase (indirect markers); myeloperoxidase; eosinophils and markers of eosinophil activation (asthma and chronic obstructive pulmonary disease overlapping, chronic obstructive pulmonary disease exacerbation); lipocalin 2; nitric oxide synthases; nitrotyrosine; 4-hydroxy-2-nonenal (4-HNE); malonaldehyde; 8-isoprostane; aldehydes; protein carbonyls/antibodies against carbonyls	Elevation compared with controls	[25]
	Superoxide dismutases; catalase; Nrf2 and antioxidant enzymes (many glutathione-mediated enzymes) related to Nrf2 regulation; glutathione; hemeoxygenase 1; sulfiredoxin; sirtuin1 (associated with oxidative stress); FOXO3 (associated with antioxidant genes)	Decrease/inactivation compared with controls (with elevation/induction of many enzymes by smoke/mild chronic obstructive pulmonary disease, but decreases with more severe disease)	[25]

**Table 2**

Clinical trials conducted for the efficacy of thiol antioxidants in chronic obstructive pulmonary disease and its exacerbations.

Study (year)	Dose	Outcome	Ref.
<i>NAC</i>			
Decramer <i>et al.</i> (2001, 2005)	600 mg/day	A reduction in lung overinflation in patients with severe COPD who were without inhaled glucocorticoids. No change of decline in FEV <sub>1</sub> . Decrease in number of exacerbations when NAC and inhaled corticosteroids were combined	[122,123]
Black <i>et al.</i> (2004)	600 mg/day	No difference in FEV <sub>1</sub> and breathlessness compared with placebo group	[127]
<i>Carbocysteine</i>			
Aylward (1974), Edwards <i>et al.</i> (1976), Miskoviti <i>et al.</i> (1982)	2.25–3 g/day	Heterogeneous results on alterations in FEV <sub>1</sub> , peak flow rate and dyspnea scores	[137–139]
Allegra <i>et al.</i> (1996)	2.7 g/day	No significant difference in baseline FEV <sub>1</sub> between the groups. Mean time to first exacerbation was significantly prolonged and significant reduction in mean days of acute respiratory illness per patient	[140]
Grillage and Barnard-Jones (1985)	750 mg/t.i.d.	No significant difference in exacerbation rate. Significant increases in peak flow from baseline in both placebo and intervention groups	[141]
Yasuda <i>et al.</i> (2006)	1.5 g/day	No significant differences in severity of COPD. Significant reduction in the number of common colds and reduction in rate of exacerbation	[142,143]
Tatsumi and Fukuchi (2007)	500 mg/t.i.d.	Consistent reduction in exacerbation frequency. No change in lung function	[136]
Zheng <i>et al.</i> (2008)	1500 mg/day	Reduction in number of exacerbations in patients with COPD	[129]

COPD: Chronic obstructive pulmonary disease; FEV<sub>1</sub> : Forced expiratory volume in 1 s; NAC: *N*-acetyl-L-cysteine; t.i.d.: Three-times daily.

**Table 3**

Cochrane reviews and meta-analyses on antioxidants in chronic obstructive pulmonary disease treatment.

Study (year)	Antioxidant	Cochrane review/meta-analysis	Study aim and outcome	Ref.
Poole <i>et al.</i> (2001, 2003)	NAC	Systematic Cochrane review of 23 randomized controlled trials	Effect of NAC and antibiotics on number of days of disability: no difference in lung function. Significant reduction in days of disability (0.65 day per patient per month) and 29% reduction in number of exacerbations	[124,125]
Stey <i>et al.</i> (2000)	NAC	Systematic Cochrane review of randomized, controlled trials; 11 of 39 retrieved trials	Use of validated score to evaluate the quality of each study: nine trials showed reduction in number of exacerbations, which five addressed improvement of symptoms compared with the patients receiving placebo	[121]
Grandjean <i>et al.</i> (2000)	NAC	Meta-analysis of published trials	Assess possible prophylactic benefits of prolonged treatment: 23% decrease in number of acute exacerbations	[126]

NAC: *N*-acetyl-L-cysteine.